

A

03/02/98
16515 U.S. PTO

DOCKET NO. : ISIS-2710

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:

Achim H. Krotz and Vasulinga T.
Ravikumar

Serial No.: Not Yet Assigned

Group Art Unit: Not Yet Assigned

Filing Date: Herewith

Examiner: Not Yet Assigned

For: IMPROVED PROCESS FOR SYNTHESIS OF OLIGONUCLEOTIDES

EXPRESS MAIL LABEL NO: EM155772072US
DATE OF DEPOSIT: January 26, 1998

Box ☒ Patent Application
☐ Provisional ☐ Design ☐ Sequence

Assistant Commissioner for Patents
Washington DC 20231

Sir:

PATENT APPLICATION TRANSMITTAL LETTER

Transmitted herewith for filing, please find

☒ A Utility Patent Application.

If this is a continuing application, please check appropriate box:

☐ continuation ☐ divisional ☐ continuation-in-part of prior application number
____/____.

- ☐ A Provisional Patent Application.
- ☐ A Design Patent Application (submitted in duplicate).

Including the following:

- ☐ Provisional Application Cover Sheet.
- ☒ New or Revised Specification, including pages 1 to 36 containing:

- ☒ Specification
- ☒ Claims
- ☒ Abstract

- ☐ A copy of earlier application Serial No. _____ Filed _____ to which no new matter has been added TOGETHER WITH a copy of the executed oath or declaration for such earlier application and all drawings and appendices. Such earlier application is hereby incorporated into the present application by reference.

- ☐ Please enter the following amendment to the Specification under the Cross Reference to Related Applications section (or create such a section) : "This Application is a ☐ continuation or ☐ divisional of Application Serial No. _____ filed _____

_____."

- ☐ Signed Statement attached deleting inventor(s) named in the prior application.

- ☐ A Preliminary Amendment.

- ☐ _____ Sheets of ☐ Formal ☐ Informal Drawings.

- ☐ Petition to Accept Photographic Drawings.

- ☐ Petition Fee

- ☒ An ☐ Executed ☒ Unexecuted Declaration or Oath and Power of Attorney.
- ☐ An Associate Power of Attorney.
- ☐ An ☐ Executed ☐ Copy of Executed Assignment of the Invention to _____
_____.
- ☐ ☐ A Recordation Form Cover Sheet.
- ☐ ☐ Recordation Fee - \$40.00.
- ☐ Priority is claimed under 35 U.S.C. § 119 of application Serial No. _____ filed _____
_____ in _____ (country).
- ☐ A Certified Copy of each of the following applications for which priority is claimed:
- ☐ is enclosed.
- ☐ has been filed in prior application Serial No. _____ filed _____.
- ☒ An ☒ Executed ☐ Unexecuted ☒ Statement Claiming Small Entity Status under 37 C.F.R. 1.9 and 1.27
- ☒ is enclosed.
- ☐ has been filed in prior application Serial No. _____ filed _____, said status is still proper and desired in present case.
- ☐ Diskette Containing DNA/Amino Acid Sequence Information.
- ☐ Statement to Support Submission of DNA/Amino Acid Sequence Information.
- ☐ Letter of Reference to Computer Readable Form.
- ☐ Information Disclosure Statement.
- ☐ Attached Form 1449.
- ☐ Copies of each of the references listed on the attached Form PTO-1449 are enclosed herewith.
- ☐ A copy of Petition for Extension of Time as filed in the prior case.

☐ Appended Material as follows: _____

☒ Return Receipt Postcard (should be specifically itemized).

☐ Other as follows: _____

FEE CALCULATION

				SMALL ENTITY		NOT SMALL ENTITY	
				RATE	FEE	RATE	FEE
PROVISIONAL APPLICATION				\$75.00	\$	\$150.00	\$
DESIGN APPLICATION				\$165.00	\$	\$330.00	\$
UTILITY APPLICATIONS BASE FEE				\$395.00	\$395	\$790.00	\$
UTILITY APPLICATION; ALL CLAIMS CALCULATED AFTER ENTRY OF ALL AMENDMENTS							
	No. Filed	No. Extra					
TOTAL CLAIMS	41 - 20 =	21		\$11 each	\$231	\$22 each	\$
INDEP. CLAIMS	2 - 3 =	0		\$41 each	\$0	\$82 each	\$
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM				\$135	\$0	\$270	\$
ADDITIONAL FILING FEE					\$231		\$
TOTAL FILING FEE DUE					\$626		\$

☒ Two (2) Checks are enclosed in the amounts of \$ 604 & \$22 totaling \$626.

☒ The Commissioner is hereby requested to grant an extension of time for the appropriate length of time, should one be necessary, in connection with this filing or any future filing submitted to the U.S. Patent and Trademark Office in the above-identified application during the pendency of this application. The Commissioner is further authorized to charge any fees related to any such extension of time to deposit account 23-3050. This sheet is provided in duplicate.

☒ The Commissioner is authorized to charge payment of the following fees and to refund any overpayment associated with this communication or during the pendency of this application to deposit account 23-3050. This sheet is provided in duplicate.

- ☐ The foregoing amount due.
- ☒ Any additional filing fees required, including fees for the presentation of extra claims under 37 C.F.R. 1.16.
- ☒ Any additional patent application processing fees under 37 C.F.R. 1.17 or 1.20(d).
- ☐ The issue fee set in 37 C.F.R. 1.18 at the mailing of the Notice of Allowance.

SHOULD ANY DEFICIENCIES APPEAR with respect to this application, including deficiencies in payment of fees, missing parts of the application or otherwise, the United States Patent and Trademark Office is respectfully requested to promptly notify the undersigned.

Date: February 26, 1998



Michael P. Straher
Registration No. 38,325

Woodcock Washburn Kurtz
Mackiewicz & Norris LLP
One Liberty Place - 46th Floor
Philadelphia PA 19103
Telephone: (215) 568-3100
Facsimile: (215) 568-3439

IMPROVED METHODS FOR SYNTHESIS OF OLIGONUCLEOTIDES

FIELD OF THE INVENTION

The present invention is directed to improved methods for synthesis of oligonucleotides and other
5 phosphorus-linked oligomers, using aromatic solvents, alkyl
aromatic solvents, halogenated aromatic solvents,
halogenated alkyl aromatic solvents, or aromatic ether
solvents. Oligomers synthesized using the methods of the
invention are useful for diagnostic reagents, research
10 reagents and in therapeutics.

BACKGROUND OF THE INVENTION

It is well known proteins are significantly
involved in many of the bodily states in multicellular
organisms, including most disease states. Such proteins,
15 either acting directly or through their enzymatic or other
functions, contribute in major proportion to many diseases
and regulatory functions in animals and man. For disease
states, classical therapeutics has generally focused upon
interactions with such proteins in efforts to moderate their
20 disease-causing or disease-potentiating functions. In newer
therapeutic approaches, modulation of the production of such
proteins is desired. By interfering with the production of
proteins, the maximum therapeutic effect might be obtained
with minimal side effects. It is the general object of such
25 therapeutic approaches to interfere with or otherwise

modulate gene expression which would lead to undesired protein formation.

One method for inhibiting specific gene expression is with the use of oligonucleotides, especially oligonucleotides which are complementary to a specific target messenger RNA (mRNA) sequence.

Transcription factors interact with double-stranded DNA during regulation of transcription. Oligonucleotides can serve as competitive inhibitors of transcription factors to modulate the action of transcription factors. Several recent reports describe such interactions (see Bielinska, A., et. al., *Science*, 1990, 250, 997-1000; and Wu, H., et. al., *Gene*, 1990, 89, 203-209).

In addition to functioning as both indirect and
15 direct regulators of proteins, oligonucleotides have also
found use in diagnostic tests. Such diagnostic tests can be
performed using biological fluids, tissues, intact cells or
isolated cellular components. As with gene expression
inhibition, diagnostic applications utilize the ability of
20 oligonucleotides to hybridize with a complementary strand of
nucleic acid. Hybridization is the sequence specific
hydrogen bonding of oligonucleotides, via Watson-Crick
and/or Hoogsteen base pairs, to RNA or DNA. The bases of
such base pairs are said to be complementary to one another.

Oligonucleotides are also widely used as research reagents. They are useful for understanding the function of many other biological molecules as well as in the preparation of other biological molecules. For example, the use of oligonucleotides as primers in polymerase chain reactions (PCR) has given rise to an expanding commercial industry. PCR has become a mainstay of commercial and research laboratories, and applications of PCR have multiplied. For example, PCR technology is used in the fields of forensics, paleontology, evolutionary studies and genetic counseling. Commercialization has led to the development of kits which assist non-molecular biology-trained personnel in applying PCR. Oligonucleotides, both

natural and synthetic, are employed as primers in PCR technology.

Laboratory uses of oligonucleotides are described generally in laboratory manuals such as *Molecular Cloning, A Laboratory Manual*, Second Ed., J. Sambrook, et al., Eds., Cold Spring Harbor Laboratory Press, 1989; and *Current Protocols In Molecular Biology*, F. M. Ausubel, et al., Eds., Current Publications, 1993. Such uses include Synthetic Oligonucleotide Probes, Screening Expression Libraries with Antibodies and Oligonucleotides, DNA Sequencing, In Vitro Amplification of DNA by the Polymerase Chain Reaction and Site-directed Mutagenesis of Cloned DNA (see Book 2 of *Molecular Cloning, A Laboratory Manual, ibid.*) and DNA-Protein Interactions and The Polymerase Chain Reaction (see Vol. 2 of *Current Protocols In Molecular Biology, ibid.*).

Oligonucleotides can be custom-synthesized for a desired use. Thus a number of chemical modifications have been introduced into oligonucleotides to increase their usefulness in diagnostics, as research reagents and as therapeutic entities. Such modifications include those designed to increase binding to a target strand (i.e. increase their melting temperatures, (T_m)); to assist in identification of the oligonucleotide or an oligonucleotide-target complex; to increase cell penetration; to stabilize against nucleases and other enzymes that degrade or interfere with the structure or activity of the oligonucleotides; to provide a mode of disruption (terminating event) once sequence-specifically bound to a target; and to improve the pharmacokinetic properties of the oligonucleotides.

Thus, it is of increasing value to prepare oligonucleotides and other phosphorus-linked oligomers for use in basic research or for diagnostic or therapeutic applications. Consequently, and in view of the considerable expense and time required for synthesis of specific oligonucleotides, there has been a longstanding effort to develop successful methodologies for the preparation of

specific oligonucleotides with increased efficiency and product purity.

Synthesis of oligonucleotides can be accomplished using both solution phase and solid phase methods.

5 Oligonucleotide synthesis via solution phase in turn can be accomplished with several coupling mechanisms. However, solution phase chemistry requires purification after each internucleotide coupling, which is labor intensive and time consuming.

10 The current method of choice for the preparation of naturally occurring oligonucleotides, as well as modified oligonucleotides such as phosphorothioate and phosphorodithioate oligonucleotides, is via solid-phase synthesis wherein an oligonucleotide is prepared on a polymer support
15 (a solid support) such as controlled pore glass (CPG); oxalyl-controlled pore glass (see, e.g., Alul, et al., *Nucleic Acids Research* 1991, 19, 1527); TENTAGEL Support, (see, e.g., Wright, et al., *Tetrahedron Letters* 1993, 34, 3373); or POROS, a polystyrene resin available from
20 Perceptive Biosystems.

Solid-phase synthesis relies on sequential addition of nucleotides to one end of a growing oligonucleotide chain. Typically, a first nucleoside (having protecting groups on any exocyclic amine functionalities present) is
25 attached to an appropriate glass bead support and activated phosphite compounds (typically nucleotide phosphoramidites, also bearing appropriate protecting groups) are added stepwise to elongate the growing oligonucleotide. The nucleotide phosphoramidites are reacted with the growing
30 oligonucleotide using "fluidized bed" technology to mix the reagents. The known silica supports suitable for anchoring the oligonucleotide are very fragile and thus cannot be exposed to aggressive mixing. Brill, W. K. D., et al. *J. Am. Chem. Soc.*, 1989, 111, 2321, disclosed a procedure
35 wherein an aryl mercaptan is substituted for the nucleotide phosphoramidite to prepare phosphorodithioate oligonucleotides on glass supports.

phosphoro-dithioate as a major product and a derivative suitable for preparation of a phosphorithioate as a minor product.

Further details of methods useful for preparing
5 oligonucleotides may be found in Sekine, M., et al., *J. Org. Chem.*, 1979, 44, 2325; Dahl, O., *Sulfur Reports*, 1991, 11, 167-192; Kresse, J., et al., *Nucleic Acids Research*, 1975, 2, 1-9; Eckstein, F., *Ann. Rev. Biochem.*, 1985, 54, 367-402; and Yau, E.K. U.S. Patent No. 5,210,264.

10 Methods for synthesizing oligonucleotides using intermediates having phosphorus-containing covalent linkages involve the protection of the 5'-hydroxyl group of a nucleoside by forming trityl or substituted trityl or triarylmethyl derivatives. The protecting groups are later
15 removed under acidic conditions to yield the free 5'-hydroxyl group. The hydroxyl group can then be further reacted to give a coupled product.

The removal of trityl and other protecting groups is generally carried out in the presence of halogenated
20 solvents such as dichloromethane or dichloroethane. However, the use of such halogenated solvents is undesirable for several reasons, particularly in relatively large scale applications such as the manufacture of oligonucleotides or analogs use as antisense agents. Consequently, there
25 remains a need for methods of synthesis of oligonucleotides which provide improved efficiency and reduced disposal problems. The present invention is directed to these, as well as other, important ends.

SUMMARY OF THE INVENTION

30 The present invention provides improved methods for the preparation of oligonucleotides.

In some preferred embodiments the present invention provides methods for the preparation of a phosphorus-linked oligomer comprising the steps of:

- 35 (a) providing a solid support;
(b) attaching a 5'-O-protected nucleoside to the

862060 22626060

solid support;

(c) deprotecting the 5'-hydroxyl of the nucleoside with a deprotecting reagent comprising a protic acid in a solvent to deprotect the 5'-hydroxyl of the nucleoside, the solvent being an aromatic solvent, an alkyl aromatic solvent, a halogenated aromatic solvent, a halogenated alkyl aromatic solvent, or an aromatic ether solvent;

(d) reacting the deprotected 5'-hydroxyl with an
5'-protected activated phosphorus compound to produce a
10 covalent linkage therebetween;

(e) oxidizing or sulfurizing the covalent linkage to form a phosphodiester, phosphorothioate, phosphorodithioate or H-phosphonate linkage;

(f) repeating steps c through e at least once for
15 subsequent couplings of additional activated phosphorus
compounds, to produce the completed phosphorus-linked
oligomer; and

(g) cleaving the oligomer from the solid support.

Some preferred embodiments of the methods of the invention further comprise the step of capping remaining reactive sites with a solution containing a capping reagent.

In some preferred embodiments, the oxidation or sulfurization step is performed after each iteration of steps (c) and (d). In other preferred embodiments, a single oxidation or sulfurization step is performed after the final iteration of steps (c) and (d).

In some preferred embodiments of the methods of the invention, solvent in step (c) is an aromatic solvent, an alkyl aromatic solvent, or an aromatic ether. In more preferred embodiments the solvent in step (c) is benzene, toluene, benzonitrile, *o*-, *m*- or *p*-xylene, mesitylene, or diphenyl ether, with benzene, toluene or *o*-, *m*- or *p*-xylene being more preferred, and toluene being particularly preferred.

35 In other preferred embodiments of the methods of the invention, the solvent in step (c) is a halogenated aromatic solvent or a halogenated alkyl aromatic solvent,

In some preferred embodiments of the methods of the invention the activated phosphorus compound is an activated mononucleotide, an activated dinucleotide, or an activated polynucleotide.

In further preferred embodiments of the method of the invention, the activated phosphorus compound is a 5'-protected nucleoside phosphoramidite or a 5'-protected activated H-phosphonate nucleoside.

In some preferred embodiments of the methods of the invention, the 5'-protecting group of the 5'-O-protected nucleoside and the 5'-protected activated phosphorus compound is independently trityl, monomethoxy trityl, dimethoxytrityl, trimethoxytrityl, 2-chlorotrityl, DATE, TBTr, Pixyl or Moxyl, with trityl, monomethoxy trityl, dimethoxy trityl, 9-phenylxanthine-9-yl (Pixyl) or 9-(p-methoxyphenyl)xanthine-9-yl MOX) being more preferred, and dimethoxy trityl being especially preferred.

In some preferred embodiments of the methods of the invention, the phosphorus-linked oligomer is a phosphodiester, phosphorothioate phosphorodithioate, or H-phosphonate oligonucleotide.

In some preferred embodiments of the methods of the invention, the protic acid is formic acid, acetic acid, chloroacetic acid, dichloroacetic acid, trichloroacetic acid, trifluoroacetic acid, benzenesulfonic acid, toluenesulfonic acid, or phenylphosphoric acid.

In further preferred embodiments the solvent in step (c) further comprises an additive, which is preferably an alcohol, with from 0% to about 30% methanol, ethanol, 2-propanol, t-butyl alcohol, t-amyl alcohol, benzyl alcohol, or 1,1,1,3,3,3-hexafluoro-2-propanol, or a mixture thereof being especially preferred.

In some further preferred embodiments, the 5'-protected activated phosphorus compound is a 5'-protected activated H-phosphonate compound; and the phosphorus-linked

[illegible][illegible]

- [illegible]

[illegible][illegible][illegible]

the invention, the solvent in step (c) is a halogenated aromatic solvent or a halogenated alkyl aromatic solvent, with chlorobenzene or benztotriufuide being especially preferred.

5 In some preferred embodiments of the methods of the invention the activated phosphorus compound is a mononucleotide phosphoramidite, a dinucleotide phosphoramidite, or a polynucleotide phosphoramidite.

In further preferred embodiments of the method of the invention, the activated phosphorus compound is a 5'-protected nucleoside phosphoramidite or a 5'-protected activated H-phosphonate nucleoside.

In some preferred embodiments of the methods of the invention, the 5'-protecting group of the 5'-O-protected nucleoside and the 5'-protected activated phosphorus compound is independently trityl, monomethoxy trityl, dimethoxytrityl, trimethoxytrityl, 2-chlorotrityl, DATE, TBTr, Pixyl or Moxyl, with trityl, monomethoxy trityl, dimethoxy trityl, 9-phenylxanthine-9-yl (Pixyl) or 9-(p-methoxyphenyl)xanthine-9-yl (MOX) being more preferred, and dimethoxy trityl being especially preferred.

In some preferred embodiments of the methods of the invention, the phosphorus-linked oligomer is a phosphodiester, phosphorothioate or phosphorodithioate oligonucleotide.

In some preferred embodiments of the methods of the invention, the protic acid is formic acid, acetic acid, chloroacetic acid, dichloroacetic acid, trichloroacetic acid, trifluoroacetic acid, benzenesulfonic acid, toluenesulfonic acid, or phenylphosphoric acid.

In further preferred embodiments the solvent in step (c) further comprises an additive, which is preferably an alcohol, with from 0% to about 30% methanol, ethanol, 2-propanol, t-butyl alcohol, t-amyl alcohol, benzyl alcohol, 35 or 1,1,1,3,3,3-hexafluoro-2-propanol, or a mixture thereof being especially preferred.

In some particularly preferred embodiments, the

solvent in step (c) is benzene, toluene, benzonitrile, o-, m- or p-xylene, mesitylene, or diphenyl ether, with toluene being especially preferred; the activated phosphite compound is a mononucleotide phosphoramidite, a dinucleotide
5 phosphoramidite, or a polynucleotide phosphoramidite; the protecting group of the 5'-O-protected nucleoside and the 5'-protected activated phosphite compound is dimethoxytrityl; the phosphorus linked oligomer is a phosphodiester, phosphorothioate or a phosphorodithioate
10 oligonucleotide; and the protic acid is dichloroacetic acid.

DETAILED DESCRIPTION

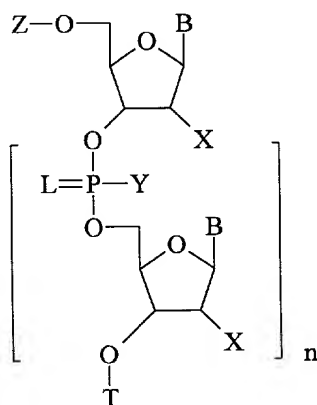
The present invention provides novel methods for the preparation of phosphorus-linked oligomers comprising the steps of:

- 15 (a) providing a solid support;
 - (b) attaching a 5'-O-protected nucleoside to the solid support;
 - (c) deprotecting the 5'-hydroxyl of the nucleoside with a deprotecting reagent comprising a protic acid in a
20 solvent to deprotect the 5'-hydroxyl of the nucleoside, the solvent being an aromatic solvent, an alkyl aromatic solvent, a halogenated aromatic solvent, a halogenated alkyl aromatic solvent, or an aromatic ether solvent;
 - (d) reacting the deprotected 5'-hydroxyl with an
25 5'-protected activated phosphorus compound to produce a covalent linkage therebetween;
 - (e) oxidizing or sulfurizing the covalent linkage to form a phosphodiester, phosphorothioate, phosphorodithioate or H-phosphonate linkage;
 - 30 (f) repeating steps c through e at least once for subsequent couplings of additional activated phosphorus compounds, to produce the completed phosphorus-linked oligomer; and
 - (g) cleaving the oligomer from the solid support.
- 35 It is generally preferable to perform a capping step after reaction of the deprotected 5'-hydroxyl with an

5'-protected activated phosphorus compound. The capping step can be performed either before or after the oxidation or sulfurization step, and is generally known to provide benefits in the prevention of shortened oligomer chains, by blocking chains that have not reacted in the coupling cycle. One representative reagent used for capping is acetic anhydride. Other suitable capping reagents and methodologies can be found in United States Patent 4,816,571.

The methods of the invention can be used for the preparation of a variety of phosphorus-linked oligonucleotide species, such as phosphodiester, phosphorothioate, phosphorodithioate and H-phosphonate oligonucleotides.

The methods of the invention can be used to prepare several types of oligonucleotides, including phosphodiesters, phosphotriesters, phosphorothioates, phosphodithioates, and H-phosphonates. These oligonucleotides are of the structure:



wherein Z is an acid labile protecting group and B is a naturally occurring nucleobase (i.e., adenine (A), cytosine (C), guanine (G), thymine (T) or uracil (U)) or any of the nucleobase analogs known in the art as described below. For the phosphodiester DNA, X = H, and B = A, C, G, or T; and

for the phosphodiester RNA, X = OH, and B = A, C, G or U. Y is H, OH, SH or alkyl, and L is O or S. T is a solid support as described below for solid phase synthesis or a base labile protecting group for solution phase synthesis.

5 In the context of the present invention, the term
"phosphorus-linked oligomer" refers to a plurality of joined
nucleobase-bearing sugar moieties connected by a linking
group having a phosphorus atom. Linking groups include
phosphodiester, phosphotriester, phosphorothioate,
10 phosphodithioate, and H-phosphonate linkages.

The structures listed above are representative of commonly synthesized phosphorus-linked oligomers, and the application of the methods of the present invention to them is illustrative, and not limiting. For example, it is known to substitute a wide variety of modifications on the above structures including base modifications, backbone modifications, phosphate modifications, sugar modifications, and 2' modifications. Recent modifications include replacing the sugar with an alternative structure which has primary and a secondary alcohol groups similar to those of ribose. As used herein, these modified compounds are included within the definition of the term "phosphorus-linked oligomers".

As used herein, the term "5'-protected activated
25 phosphorus compound" is intended to denote a mono-, di- or
polynucleoside species that has an activated phosphorus group
at its 3'-terminus, and bears a 5'-hydroxyl protecting
group. The activated phosphorus group is one that is known
in the art to undergo a coupling reaction with the
30 deprotected 5'-hydroxyl of a growing oligomeric chain
according to standard synthetic methodologies, such as, for
example, the phosphoramidite, phosphotriester and H-
phosphonate synthetic methods. See for example Caruthers
U.S. Patents Nos. 4,415,732; 4,458,066; 4,500,707;
35 4,668,777; 4,973,679; and 5,132,418; and Koster U.S. Patents
Nos. 4,725,677 and Re. 34,069; Sekine, M., et al., *J. Org.
Chem.*, 1979, 44, 2325; Dahl, O., *Sulfur Reports*, 1991, 11,

167-192; Kresse, J., et al. ., *Nucleic Acids Research*, 1975, 2, 1-9; Eckstein, F., *Ann. Rev. Biochem.*, 1985, 54, 367-402; and Yau, E.K. U.S. Patent No. 5,210,264, and *Oligonucleotides and Analogues A Practical Approach*,

5 Eckstein, F. Ed., IRL Press, New York, 1991, each of the disclosures of which are hereby incorporated by reference in their entirety. Thus, activated phosphorus groups include groups of formula $-P(-O-Pr)-N(i-pr)_2$ where Pr is a phosphorus protecting group useful in phosphoramidite synthesis, such
10 β -cyanoethyl.

In accordance with the methods of the invention, a 5'-O-protected nucleoside synthon is first attached to a solid support. Solid supports are substrates which are capable of serving as the solid phase in solid phase
15 synthetic methodologies, such as those described in Caruthers U.S. Patents Nos. 4,415,732; 4,458,066; 4,500,707; 4,668,777; 4,973,679; and 5,132,418; and Koster U.S. Patents Nos. 4,725,677 and Re. 34,069. Linkers are known in the art as short molecules which serve to connect a solid support to
20 functional groups (e.g., hydroxyl groups) of initial synthon molecules in solid phase synthetic techniques. One such linker is a succinamide linker. Other suitable linkers are disclosed in, for example, *Oligonucleotides And Analogues A Practical Approach*, Ekstein, F. Ed., IRL Press, N.Y, 1991,
25 Chapter 1, pages 1-23.

Solid supports according to the invention include those generally known in the art to be suitable for use in solid phase methodologies, including, for example, controlled pore glass (CPG), oxalyl-controlled pore glass
30 (see, e.g., Alul, et al., *Nucleic Acids Research* 1991, 19, 1527), TentaGel Support (an aminopolyethyleneglycol derivatized support (see, e.g., Wright, et al., *Tetrahedron Letters* 1993, 34, 3373)) and Poros (a copolymer of polystyrene/divinylbenzene).

35 In accordance with the methods of the invention, after the initial nucleoside synthon is attached to the solid support, the 5'-hydroxyl of the nucleoside deprotected

Heretofore, deblocking of 5'-hydroxyl groups has been accomplished using such protic acids in a halogenated alkyl solvent such as dichloromethane or dichloroethane. However the use of such halogenated alkyl solvents is greatly disadvantageous because they are not easily disposed of (and therefore expensive to use) because of the environmental hazard they pose. For example, methylene chloride has been classified as a carcinogen by OSHA, and such low boiling solvents require a relatively large investment in recycling equipment.

It has been discovered in accordance with the present invention that deblocking reactions can be performed in solvents other than halogenated alkyl solvents. Thus, in accordance with preferred embodiments of the invention, deprotection (i.e., deblocking) of the 5'-O- protecting group is accomplished using a protic acid such as formic acid, acetic acid, chloroacetic acid, dichloroacetic acid, trichloroacetic acid, trifluoroacetic acid, benzenesulfonic acid, toluenesulfonic acid, or phenylphosphoric acid, in an aromatic solvent, an alkyl aromatic solvent, a halogenated aromatic solvent, a halogenated alkyl aromatic solvent, or an aromatic ether solvent.

25 In more preferred embodiments, the solvent is benzene, toluene, benzonitrile, *o*-, *m*- or *p*-xylene, mesitylene, or diphenyl ether. In still more preferred embodiments the solvent is benzene, toluene or *o*-, *m*- or *p*-xylene, with toluene being particularly preferred.

30 In some preferred embodiments, the solvent is high
boiling; i.e., it has a boiling point greater than about
60°C. Such solvents are additionally advantageous in that
they do not require a substantial investment in recycling
equipment that meets stringent environmental regulations
35 required by OSHA for lower boiling solvents.

As used herein, the term alkyl includes but is not limited to straight chain, branch chain, and alicyclic

[illegible]

5 incorporated by reference in its entirety.

10 where the substituents include halogen, nitro, or
hydroxymethyl.

15 known to be useful as solvents in the art. These include
for example, toluene, *o*-, *m*- or *p*-xylene, and mesitylene.
Also included within the definition of "alkyl aromatic
solvent" are liquid aromatic ring compounds that contain
cyano groups, such as benzonitrile.

20 Halogens include fluorine, chlorine, bromine and
iodine.

25 denotes an aromatic compound having at least one heteroatom (i.e., an atom other than carbon) in the aromatic ring.

30 produce a covalent linkage therebetween. In some preferred
embodiments wherein the activated phosphorus compound is a
phosphoramidite, a phosphite linkage is produced. In other
preferred embodiments, such as in H-phosphonate chemistries,
the activated phosphorus group has the formula
35 $-O-HP(=O)-O^-$, and reacts with a free 3'-hydroxyl of the
growing oligonucleotide chain in the presence of an
activating agent such as pivaloyl chloride to produce an

H-phosphonate linkage.

In some preferred embodiments, a sulfurization step or oxidation step is performed after each deprotection-coupling cycle. However, if desired, oxidation or sulfurization can be performed in a single step at the end of the iterative synthesis.

Useful oxidizing agents according to the present invention include iodine, t-butyl hydroperoxide, or other oxidizing reagents known in the art.

Sulfurizing agents used during oxidation to form phosphorothioate linkages include Beaucage reagent (see e.g. Iyer, R.P., et al. ., *J. Chem. Soc.*, 1990, 112, 1253-1254, and Iyer, R.P., et al., *J. Org. Chem.*, 1990, 55, 4693-4699); tetraethylthiuram disulfide (see e.g., Vu, H., Hirschbein, B.L., *Tetrahedron Lett.*, 1991, 32, 3005-3008); dibenzoyl tetrasulfide (see e.g., Rao, M.V., et al. ., *Tetrahedron Lett.*, 1992, 33, 4839-4842); di(phenylacetyl)disulfide (see e.g., Kamer, P.C.J., *Tetrahedron Lett.*, 1989, 30, 6757-6760); 1,2,4-dithiiazoline-5-one (DtsNH) and 3-ethoxy-1,2,4-dithiiazoline-5-one (EDITH) and (see Xu et al., *Nucleic Acids Research*, 1996, 24, 3643-3644 and Xu et al., *Nucleic Acids Research*, 1996, 24, 1602-1607); thiophosphorus compounds such as those disclosed in U.S. patent No. 5,292,875 to Stec et al., and U.S. patent No. 5,151,510 to Stec et al., disulfides of sulfonic acids, such as those disclosed in Efimov et al., *Nucleic Acids Research*, 1995, 23, 4029-4033, sulfur, sulfur in combination with ligands like triaryl, trialkyl, triaralkyl, or trialkaryl phosphines.

The deprotection and coupling steps, and, optionally, oxidation or sulfurization steps, are repeated using mono-, di- or polymeric activated synthons until the desired base sequence is achieved. The completed oligomer is then cleaved from the solid support. Cleavage is achieved by any of the standard methods in the art, such as, for example, with concentrated ammonium hydroxide.

The methods of the present invention can be used

for the synthesis of phosphorus-linked oligonucleotides having both naturally occurring and non-naturally occurring constituent groups. For example, the present invention can be used to synthesize phosphodiester, phosphorothioate, 5 phosphorodithioate, and H-phosphonate oligomers having naturally occurring pentose sugar components such as ribose and deoxyribose, and their substituted derivatives, as well as other sugars known to substitute therefor in oligonucleotide analogs.

10 The constituent sugars and nucleosidic bases of the phosphorus-linked oligonucleotides can be naturally occurring or non-naturally occurring. Non-naturally occurring sugars and nucleosidic bases are typically structurally distinguishable from, yet functionally inter- 15 changeable with, naturally occurring sugars (e.g. ribose and deoxyribose) and nucleosidic bases (e.g., adenine, guanine, cytosine, thymine). Thus, non-naturally occurring nucleobases and sugars include all such structures which mimic the structure and/or function of naturally occurring 20 species, and which aid in the binding of the oligonucleotide to a target, or which otherwise advantageously contribute to the properties of the oligonucleotide.

 The methods of the invention are amenable to the synthesis of phosphorus-linked oligomers having a variety of 25 substituents attached to their 2'-positions. These include, for example, halogens, O-alkyl, O-alkylamino, O-alkylalkoxy, protected O-alkylamino, O-alkylaminoalkyl, O-alkyl imidazole, and polyethers of the formula (O-alkyl)_m, where m is 1 to about 10. Preferred among these polyethers are 30 linear and cyclic polyethylene glycols (PEGs), and (PEG)-containing groups, such as crown ethers and those which are disclosed by Ouchi, et al., *Drug Design and Discovery* 1992, 9, 93, Ravasio, et al., *J. Org. Chem.* 1991, 56, 4329, and Delgado et. al., *Critical Reviews in Therapeutic Drug* 35 *Carrier Systems* 1992, 9, 249. Further sugar modifications are disclosed in Cook, P.D., *supra*. Fluoro, O-alkyl, O-alkylamino, O-alkyl imidazole, O-alkylaminoalkyl, and alkyl

8520E0" 2/62E060

5

15

20

25

30

35

2620297 030333
2620297 030333

The methods of the present invention use labile protecting groups to protect various functional moieties during synthesis. Protecting groups are used ubiquitously in standard oligonucleotide synthetic regimes for protection of several different types of functionality. In general, protecting groups render chemical functionality inert to specific reaction conditions and can be appended to and removed from such functionality in a molecule without substantially damaging the remainder of the molecule. See, e.g., Green and Wuts, *Protective Groups in Organic Synthesis*, 2d edition, John Wiley & Sons, New York, 1991. Representative protecting groups useful to protect nucleotides during synthesis include base labile protecting groups and acid labile protecting groups. Base labile protecting groups are used to protect the exocyclic amino groups of the heterocyclic nucleobases. This type of protection is generally achieved by acylation. Two commonly used acylating groups for this purpose are benzoylchloride and isobutyrylchloride. These protecting groups are stable to the reaction conditions used during oligonucleotide synthesis and are cleaved at approximately equal rates during the base treatment at the end of synthesis.

In some preferred embodiments, the 5'-protected activated phosphorus compound is a nucleoside phosphoramidite. Phosphoramidites of numerous nucleosides and derivatized solid supports are commercially available through various companies (e.g. Applied Biosystems Inc., Millipore Corp.). The amino moiety of such phosphordiamidites can be selected from various amines presently used for phosphoramidites in standard oligonucleotide synthesis. These include both aliphatic and heteroalkyl amines. One preferred amino group is diisopropylamino. Other examples of suitable amines as are described in various United States patents, principally those to M. Caruthers and associates. These include United States patents 4,668,777; 4,458,066; 4,415,732; and 4,500,707; all of which are herein incorporated by

reference.

Hydroxyl protecting groups typically used in oligonucleotide synthesis may be represented by the following structure:



wherein each of R_1 , R_2 and R_3 is an unsubstituted or mono-substituted aryl or heteroaryl group selected from phenyl, naphthyl, anthracyl, and five or six membered heterocyclic rings with a single heteroatom selected from N, O and S, or
10 two N heteroatoms, including quinolyl, furyl, and thienyl; where the substituent is selected from halo (i.e., F, Cl, Br, and I), nitro, C_1 - C_4 -alkyl or alkoxy, and aryl, aralkyl and cycloalkyl containing up to 10 carbon atoms; and wherein R_2 and R_3 may each also be C_1 - C_4 -alkyl or aralkyl or
15 cycloalkyl containing up to 10 carbon atoms.

In preferred embodiments of the invention, the 5'-protecting group is trityl, monomethoxy trityl, dimethoxytrityl, trimethoxytrityl, 2-chlorotrityl, DATE, TBTr, Pixyl or Moxyl, with trityl, monomethoxy trityl,
20 dimethoxy trityl, 9-phenylxanthine-9-yl (Pixyl) or 9-(p-methoxyphenyl)xanthine-9-yl (MOX) being more preferred, and with dimethoxy trityl being especially preferred.

In some preferred embodiments, the present invention provides methods for the preparation of a
25 phosphorus-linked oligomer comprising the steps of:

- a) providing a solid support;
- b) attaching a 5'-O-protected nucleoside to the solid support;
- c) contacting the protected 5'-hydroxyl of the
30 nucleoside with a deprotecting reagent comprising a protic acid in a solvent to deprotect the 5'-hydroxyl of the nucleoside, the solvent being an aromatic solvent, an alkyl

862030 2252050

aromatic solvent, a halogenated aromatic solvent, a halogenated alkyl aromatic solvent, or an aromatic ether solvent;

d) reacting the deprotected 5'-hydroxyl with a 5'-protected activated phosphite compound to produce a phosphite linkage;

e) oxidizing or sulfurizing the phosphite linkage to form a phosphodiester, phosphorothioate, or phosphorodithioate linkage;

f) repeating steps c through e at least once for subsequent couplings of additional activated phosphite compounds, to produce the completed phosphorus-linked oligomer; and

g) cleaving the oligomer from the solid support.

As used herein, the term "activated phosphite compound" is intended to include mono-, di- and polynucleoside phosphoramidites.

As used herein, the term "5'-protected activated phosphite compound" is intended to include a 5'-protected mono-, di- and polynucleoside phosphoramidite, as is used in standard solid phase oligonucleotide synthesis.

In some preferred embodiments of the invention the phosphordiamidite is activated to nucleophilic attack by the 5' hydroxyl by use of an activating agent. It is believed that the activating agent displaces one of the amino groups from the phosphordiamidite, thereby rendering the phosphorus of the phosphordiamidite more susceptible to nucleophilic attack by the 5' hydroxyl group of the growing nucleotide chain. Any activating agent that can activate the phosphorous to nucleophilic attack without interacting with the growing nucleotide chain may be suitable for use with the present invention. One preferred activating agent is tetrazole. Some commonly used commercially available activating agents are thiotetrazole, nitrotetrazole, and N,N-diisopropylaminohydro-tetrazolide. Other suitable activating agents are also disclosed in the above incorporated patents as well as in United States patent

4,725,677 and in Berner, S., Muhlegger, K., and Seliger, H.,
Nucleic Acids Research 1989, 17:853; Dahl, B.H., Nielsen, J.
and Dahl, O., *Nucleic Acids Research* 1987, 15:1729; and
Nielson, J. Marugg, J.E., Van Boom, J.H., Honnens, J.,
5 Taagaard, M. and Dahl, O., *J. Chem. Research* 1986, 26, all
of which are herein incorporated by reference.

Phosphorus linked oligomers produced by the methods
of the invention will preferably be hybridizable to a
specific target oligonucleotide. Preferably, the phosphorus
10 linked oligomers produced by the methods of the invention
comprise from about 1 to about 100 monomer subunits. It is
more preferred that such compounds comprise from about 10 to
about 30 monomer subunits, with 15 to 25 monomer subunits
being particularly preferred.

15 As will be recognized, the process steps of the
present invention need not be performed any particular
number of times or in any particular sequence. Additional
objects, advantages, and novel features of this invention
will become apparent to those skilled in the art upon
20 examination of the following examples thereof, which are not
intended to be limiting.

Example 1

Synthesis of 5'-TTTTTT-3' phosphorothioate heptamer

50 milligram (2 μ mole) of 5'-O-
25 dimethoxytritylthymidine bound to CPG (controlled pore
glass) through an ester linkage is taken up in a glass
reactor, and a toluene solution of 3% dichloroacetic acid
(volume/volume) is added to deprotect the 5'-hydroxyl group.
The product is washed with acetonitrile. Then, a 0.2 M
30 solution of 5'-O-(4,4'-dimethoxytrityl)thymidine-3'-O-(2-
cyanoethyl N,N-diisopropylphosphoramidite) in acetonitrile
and a 0.4 M solution of 1H-tetrazole in acetonitrile is
added, and allowed to react at room temperature for 5
minutes. The product is washed with acetonitrile, and then a
35 0.2 M solution of phenylacetyl disulfide in acetonitrile:3-

picoline (1:1 v/v) is added and allowed to react at room temperature for 3 minutes. This sulfurization step is repeated one more time for 3 minutes. The support is washed with acetonitrile, and then a solution of acetic anhydride/lutidine/THF (1:1:8), and N-methyl imidazole/THF is added to cap any unreacted 5'-hydroxyl group. The product is washed with acetonitrile.

This complete cycle is repeated five more times to produce the completely protected thymidine heptamer. The carrier containing the compound is treated with 30% aqueous ammonium hydroxide solution for 90 minutes at room temperature. The aqueous solution is filtered, and concentrated under reduced pressure to give a phosphorothioate heptamer, TTTTTTT.

Example 2

Synthesis of 5'-d(GACT)-3' phosphorothioate tetramer

50 milligram (2 μ mole) of 5'-O-dimethoxytritylthymidine bound to CPG (controlled pore glass) through an ester linkage is taken up in a glass reactor, and a toluene solution of 3% dichloroacetic acid in toluene (volume/volume) is added to deprotect the 5'-hydroxyl group. The product is washed with acetonitrile. Then, a 0.2 M solution of 5'-O-(4,4'-dimethoxytrityl)thymidine-3'-O-(2-cyanoethyl N,N-diisopropylphosphoramidite) in acetonitrile and a 0.4 M solution of 1H-tetrazole in acetonitrile is added, and allowed to react at room temperature for 5 minutes. The product is washed with acetonitrile, and then a 0.2 M solution of phenylacetyl disulfide in acetonitrile:3-picoline (1:1 v/v) is added and allowed to react at room temperature for 3 minutes. This sulfurization step is repeated one more time for 3 minutes. The support is washed with acetonitrile and then a solution of acetic anhydride/lutidine/THF (1:1:8), and N-methyl imidazole/THF

is added to cap the unreacted 5'-hydroxyl group. The product is washed with acetonitrile.

A solution of 3% dichloroacetic acid in toluene (volume/volume) is added to deprotect the 5'-hydroxyl group. The product is washed with acetonitrile. Then, a 0.2 M solution of N⁴-benzoyl-5'-O-(4,4'-dimethoxytrityl)-2'-deoxycytidine-3'-O-(2-cyanoethyl N,N-diisopropylphosphoramidite) in acetonitrile and a 0.4 M solution of 1H-tetrazole in acetonitrile is added, and allowed to react at room temperature for 5 minutes. The product is washed with acetonitrile, and then a 0.2 M solution of phenylacetyl disulfide in acetonitrile:3-picoline (1:1 v/v) is added and allowed to react at room temperature for 3 minutes. This sulfurization step is repeated one more time for 3 minutes. The support is washed with acetonitrile and then a solution of acetic anhydride/lutidine/THF (1:1:8), and N-methyl imidazole/THF is added to cap any unreacted 5'-hydroxyl group. The product is washed with acetonitrile.

20 A solution of 3% dichloroacetic acid in toluene
(volume/volume) is added to deprotect the 5'-hydroxyl group.
The product is washed with acetonitrile. Then, a 0.2 M
solution of N⁶-benzoyl-5'-O-(4,4'-dimethoxytrityl)-2'-
deoxyadenosine-3'-O-(2-cyanoethyl N,N-
25 diisopropylphosphoramidite) in anhydrous acetonitrile and a
0.4 M solution of 1H-tetrazole in acetonitrile is added, and
allowed to react at room temperature for 5 minutes. The
product is washed with acetonitrile, and then a 0.2 M
solution of phenylacetyl disulfide in acetonitrile:3-
30 picoline (1:1 v/v) is added and allowed to react at room
temperature for 3 minutes. This sulfurization step is
repeated one more time for 3 minutes. The support is washed
with acetonitrile and then a solution of acetic
anhydride/lutidine/THF (1:1:8), and N-methyl imidazole/THF
35 is added to cap the unreacted 5'-hydroxyl group. The product

is washed with acetonitrile.

A solution of 3% dichloroacetic acid in toluene (volume/volume) is added to deprotect the 5'-hydroxyl group. The product is washed with acetonitrile. Then, a 0.2 M solution of N²-isobutyryl-5'-O-(4,4'-dimethoxytrityl)-2'-deoxyguanosine-3'-O-(2-cyanoethyl N,N-diisopropylphosphoramidite) in acetonitrile and a 0.4 M solution of 1H-tetrazole in acetonitrile is added, and allowed to react at room temperature for 5 minutes. The product is washed with acetonitrile, and then a 0.2 M solution of phenylacetyl disulfide in acetonitrile:3-picoline (1:1 v/v) is added and allowed to react at room temperature for 3 minutes. This sulfurization step is repeated one more time for 3 minutes. The support is washed with acetonitrile and then a solution of acetic anhydride/lutidine/THF (1:1:8), and N-methyl imidazole/THF is added to cap any unreacted 5'-hydroxyl group. The product is washed with acetonitrile.

The carrier containing the compound is treated with 30% aqueous ammonium hydroxide solution for 90 minutes at room temperature and then incubated at 55 °C for 24 hour. The aqueous solution is filtered, concentrated under reduced pressure to give a phosphorothioate tetramer of 5'-dG-dA-dC-T-3'.

Example 3

Synthesis of fully-modified 5'-d(TCC-CGC-CTG-TGA-CAT-GCA-TT)-3' phosphorothioate 20-mer

The synthesis of the above sequence was performed on a Pharmacia OligoPilot II Synthesizer on a 620 μmole scale using the cyanoethyl phosphoramidites and Pharmacia's primary support. Detritylation was performed using 3% dichloroacetic acid in toluene (volume/volume). Sulfurization was performed using a 0.2 M solution of phenylacetyl disulfide in acetonitrile:3-picoline (1:1 v/v)

for 2 minutes. At the end of synthesis, the support was washed with acetonitrile, treated with 30% aqueous ammonium hydroxide solution for 90 minutes at room temperature and then incubated at 55 C for 24 hours to give the product.

5 **Example 4**

Synthesis of fully-modified 5'-d(GCC-CAA-GCT-GGC-ATC-CGT-CA)-3' phosphorothioate 20-mer

The synthesis of the above sequence was performed on a Pharmacia OligoPilot II Synthesizer on a 620 μ mole scale using the cyanoethyl phosphoramidites and Pharmacia's primar support. Detritylation was performed using 3% dichloroacetic acid in toluene (volume/volume). Sulfurization was performed using a 0.2 M solution of phenylacetyl disulfide in acetonitrile:3-picoline (1:1 v/v) for 2 minutes. At the end of synthesis, the support was washed with acetonitrile, treated with 30% aqueous ammonium hydroxide solution for 90 minutes at room temperature and then incubated at 55 C for 24 hours to give the product.

Example 5

20 Synthesis of fully-modified 5'-d(GCG-TTT-GCT-CTT-CTT-CTT-GCG)-3' phosphorothioate 21-mer

The synthesis of the above sequence was performed on a Pharmacia OligoPilot II Synthesizer on a 620 μ mole scale using the cyanoethyl phosphoramidites and Pharmacia's primar support. Detritylation was performed using 3% dichloroacetic acid in toluene (volume/volume). Sulfurization was performed using a 0.2 M solution of phenylacetyl disulfide in acetonitrile:3-picoline (1:1 v/v) for 2 minutes. At the end of synthesis, the support was washed with acetonitrile, treated with 30% aqueous ammonium hydroxide solution for 90 minutes at room temperature and then incubated at 55 C for 24 hours to give the product.

Example 6**Synthesis of fully-modified 5'-d(GTT-CTC-GCT-GGT-GAG-TTT-CA)-3' phosphorothioate 20-mer**

The synthesis of the above sequence was performed on a Pharmacia OligoPilot II Synthesizer on a 620 μ mole scale using the cyanoethyl phosphoramidites and Pharmacia's primar support. Detritylation was performed using 3% dichloroacetic acid in toluene (volume/volume). Sulfurization was performed using a 0.2 M solution of phenylacetyl disulfide in acetonitrile:3-picoline (1:1 v/v) for 2 minutes. At the end of synthesis, the support was washed with acetonitrile, treated with 30% aqueous ammonium hydroxide solution for 90 minutes at room temperature and then incubated at 55 C for 24 hours to give the product.

Example 7**Synthesis of fully-modified 5'-d(TCC-CGC-CTG-TGA)-2'-methoxyethyl-(CAT-GCA-TT)-3' phosphorothioate 20-mer**

The synthesis of the above sequence was performed on a Milligen 8800 Synthesizer on a 282 μ mole scale using cyanoethyl phosphoramidites and Pharmacia's primar support. Detritylation was performed using 3% dichloroacetic acid in toluene (volume/volume). Sulfurization was performed using a 0.4 M solution of phenylacetyl disulfide in acetonitrile:3-picoline (1:1 v/v) for 6 minutes. At the end of synthesis, the support was washed with acetonitrile, treated with 30% aqueous ammonium hydroxide solution for 90 minutes at room temperature and then incubated at 55 C for 24 hours to give the product.

Example 8**Synthesis of fully-modified 5'-d(TCC-CGC-CTG-TGA)-2'-methoxyethyl-(CAT-GCA-TT)-3' phosphorothioate 20-mer**

The synthesis of the above sequence was performed on a

Pharmacia OligoPilot II Synthesizer on a 250 μ mole scale using the cyanoethyl phosphoramidites and Pharmacia's primar support. Detritylation was performed using 3% dichloroacetic acid in toluene (volume/volume). Sulfurization was
5 performed using a 0.4 M solution of phenylacetyl disulfide in acetonitrile:3-picoline (1:1 v/v) for 6 minutes. At the end of synthesis, the support was washed with acetonitrile, treated with 30% aqueous ammonium hydroxide solution for 90 minutes at room temperature and then incubated at 55 C for 24
10 hours to give the product.

It is intended that each of the patents, applications, printed publications, and other published documents mentioned or referred to in this specification be herein incorporated by reference in their entirety.

15 Those skilled in the art will appreciate that numerous changes and modifications may be made to the preferred embodiments of the invention and that such changes and modifications may be made without departing from the spirit of the invention. It is therefore intended that the
20 appended claims cover all such equivalent variations as fall within the true spirit and scope of the invention.

852020 2252050

WHAT IS CLAIMED IS:

1. A method for the preparation of a phosphorus-linked oligomer comprising the steps of:

- (a) providing a solid support;
- 5 (b) attaching a 5'-O-protected nucleoside to the solid support;
- (c) deprotecting the 5'-hydroxyl of the nucleoside with a deprotecting reagent comprising a protic acid in a solvent to deprotect the 5'-hydroxyl of the nucleoside, the
10 solvent being an aromatic solvent, an alkyl aromatic solvent, a halogenated aromatic solvent, a halogenated alkyl aromatic solvent, or an aromatic ether solvent;
- (d) reacting the deprotected 5'-hydroxyl with an 5'-protected activated phosphorus compound to produce a
15 covalent linkage therebetween;
- (e) oxidizing or sulfurizing the covalent linkage to form a phosphodiester, phosphorothioate, phosphorodithioate or H-phosphonate linkage;
- (f) repeating steps c through e at least once for
20 subsequent couplings of additional activated phosphorus compounds, to produce the completed phosphorus-linked oligomer; and
- (g) cleaving the oligomer from the solid support.

2. The method of claim 1 further comprising the step
25 of capping remaining reactive sites with a solution containing a capping reagent.

3. The method of claim 1 wherein the oxidation or sulfurization step is performed after each iteration of steps (c) and (d).
30

4. The method of claim 1 wherein the oxidation or sulfurization step is performed after the final iteration of steps (c) and (d).

5. The method of claim 1 wherein the solvent in step

09032972.030299
862020 2/26/2006

(c) is an aromatic solvent, an alkyl aromatic solvent, or an aromatic ether.

6. The method of claim 1 wherein the solvent in step (c) is benzene, toluene, benzonitrile, *o*-, *m*- or *p*-xylene, mesitylene, or diphenyl ether.

7. The method of claim 6 wherein the solvent in step (c) is benzene, toluene or *o*-, *m*- or *p*-xylene.

8. The method of claim 7 wherein the solvent in step (c) is toluene.

9. The method of claim 1 wherein the solvent in step (c) is a halogenated aromatic solvent or a halogenated alkyl aromatic solvent.

10. The method of claim 9 wherein the solvent in step (c) is chlorobenzene or benzotrifluoride.

11. The method of claim 1 wherein the activated phosphorus compound is an activated mononucleotide, an activated dinucleotide, or an activated polynucleotide.

12. The method of claim 1 wherein the activated phosphorus compound is a 5'-protected nucleoside phosphoramidite or a 5'-protected activated H-phosphonate nucleoside.

13. The method of claim 1 wherein the protecting group of the 5'-O-protected nucleoside and the 5'-protected activated phosphite compound is independently trityl, monomethoxy trityl, dimethoxytrityl, trimethoxytrityl, 2-chlorotrityl, DATE, TBTr, 9-phenylxanthine-9-yl (Pixyl) or 9-(*p*-methoxyphenyl)xanthine-9-yl (MOX).

14. The method of claim 13 wherein the protecting

group of the 5'-O-protected nucleoside and the 5'-protected activated phosphite compound is independently trityl, monomethoxy trityl, dimethoxy trityl, 9-phenylxanthine-9-yl (Pixyl) or 9-(p-methoxyphenyl)xanthine-9-yl.

- 5 15. The method of claim 14 wherein the protecting group of the 5'-O-protected nucleoside and the 5'-protected activated phosphite compound is dimethoxytrityl.

16. The method of claim 1 wherein the phosphorus-linked oligomer is a phosphodiester, phosphorothioate
10 phosphorodithioate, or H-phosphonate oligonucleotide.

17. The method of claim 1 wherein the protic acid is formic acid, acetic acid, chloroacetic acid, dichloroacetic acid, trichloroacetic acid, trifluoroacetic acid, benzenesulfonic acid, toluenesulfonic acid, or
15 phenylphosphoric acid.

18. The method of claim 1 wherein the solvent in step (c) further comprises an additive.

19. The method of claim 18 wherein the additive to the solvent in step (c) is an alcohol.

- 20 20. The method of claim 19 wherein the alcohol additive to the solvent in step (c) is from 0% to about 30% methanol, ethanol, 2-propanol, t-butyl alcohol, t-amyl alcohol, benzyl alcohol, or 1,1,1,3,3,3-hexafluoro-2-propanol, or a mixture thereof.

- 25 21. A method for the preparation of a phosphorus-linked oligomer comprising the steps of:
- a) providing a solid support;
 - b) attaching a 5'-O-protected nucleoside to the solid support;
 - 30 c) contacting the protected 5'-hydroxyl of the

5 halogenated alkyl aromatic solvent, or an aromatic ether solvent;

d) reacting the deprotected 5'-hydroxyl with a 5'-protected activated phosphite compound to produce a phosphite linkage;

10 e) oxidizing or sulfurizing the phosphite linkage to
form a phosphodiester, phosphorothioate, or
phosphorodithioate linkage;

f) repeating steps c through e at least once for subsequent couplings of additional activated phosphite
15 compounds, to produce the completed phosphorus-linked
oligomer; and

α) cleaving the oligomer from the solid support.

22. The method of claim 21 further comprising the step
of capping remaining reactive sites with a solution
20 containing a capping reagent.

23. The method of claim 21 wherein the solvent in step (c) is an aromatic solvent, an alkyl aromatic solvent, or an aromatic ether.

24. The method of claim 23 wherein the solvent in step
25 (c) is benzene, toluene, benzonitrile, o-, m- or p-xylene,
mesitylene, or diphenyl ether.

25. The method of claim 24 wherein the solvent in step (c) is benzene, toluene or o-, m- or p-xylene.

26. The method of claim 25 wherein the solvent in step
30 (c) is toluene.

27. The method of claim 21 wherein the solvent in step

(c) is a halogenated aromatic solvent or a halogenated alkyl aromatic solvent.

28. The method of claim 27 wherein the solvent in step (c) is chlorobenzene or benzotrifluoride.

5 29. The method of claim 21 wherein the activated phosphite compound is a mononucleotide phosphoramidite, a dinucleotide phosphoramidite, or a polynucleotide phosphoramidite.

10 30. The method of claim 21 wherein the protecting group of the 5'-O-protected nucleoside and the 5'-protected activated phosphite compound is independently trityl, monomethoxy trityl, dimethoxytrityl, trimethoxytrityl, 2-chlorotrityl, DATE, TBTr, 9-phenylxanthine-9-yl (Pixyl) or 9-(p-methoxyphenyl)xanthine-9-yl (MOX).

15 31. The method of claim 30 wherein the protecting group of the 5'-O-protected nucleoside and the 5'-protected activated phosphite compound is independently trityl, monomethoxy trityl, dimethoxy trityl, 9-phenylxanthine-9-yl (Pixyl) or 9-(p-methoxyphenyl)xanthine-9-yl.

20 32. The method of claim 31 wherein the protecting group of the 5'-O-protected nucleoside and the 5'-protected activated phosphite compound is dimethoxytrityl.

25 33. The method of claim 21 wherein the phosphorus-linked oligomer is a phosphodiester, phosphorothioate or a phosphorodithioate oligonucleotide.

30 34. The method of claim 21 wherein the protic acid is formic acid, acetic acid, chloroacetic acid, dichloroacetic acid, trichloroacetic acid, trifluoroacetic acid, benzenesulfonic acid, toluenesulfonic acid, or phenylphosphoric acid.

35. The method of claim 21 wherein the solvent in step (c) further comprises an additive.

36. The method of claim 35 wherein the additive to the solvent in step (c) is an alcohol.

5 37. The method of claim 36 wherein the alcohol additive to the solvent in step (c) is from 0% to about 30% methanol, ethanol, 2-propanol, *t*-butyl alcohol, *t*-amyl alcohol, benzyl alcohol, or 1,1,1,3,3,3-hexafluoro-2-propanol, or a mixture thereof.

10 38. The method of claim 22 wherein the solvent in step (c) is benzene, toluene, benzonitrile, *o*-, *m*- or *p*-xylene, mesitylene, or diphenyl ether; the activated phosphite compound is a mononucleotide phosphoramidite, a dinucleotide phosphoramidite, or a polynucleotide phosphoramidite; the
15 protecting group of the 5'-O-protected nucleoside and the 5'-protected activated phosphite compound is dimethoxytrityl; the phosphorus linked oligomer is a phosphodiester, phosphorothioate or a phosphorodithioate oligonucleotide; and the protic acid is dichloroacetic acid.

20 39. The method of claim 38 wherein the solvent in step (c) is toluene.

40. The method of claim 39 wherein the activated phosphite compound is a mononucleotide phosphoramidite.

25 41. The method of claim 1 wherein the 5'-protected activated phosphorus compound is a 5'-protected activated H-phosphonate compound; and the phosphorus-linked oligomer is a H-phosphonate oligonucleotide.

09032972 030299

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to be material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Application Serial No.	Filing Date	Status
------------------------	-------------	--------

--	--	--

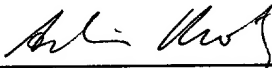

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: **John W. Caldwell** and **Michael P Straher**, Registration Nos. **28,937** and **38,325** of the firm of **WOODCOCK WASHBURN KURTZ MACKIEWICZ & NORRIS LLP**, One Liberty Place - 46th Floor, Philadelphia, Pennsylvania 19103, and **Herb Boswell**, **Laurel Bernstein** and **Andrew E. Granston**, Registration Nos. **27,311**, **37,280** and **38,473**, of **ISIS Pharmaceuticals**, 2292 Faraday Avenue, Carlsbad, California 92008.

Address all telephone calls and correspondence to:

Michael P. Straher, Esq.
WOODCOCK WASHBURN KURTZ MACKIEWICZ & NORRIS LLP
One Liberty Place - 46th Floor
Philadelphia, PA 19103
Telephone No. **215-568-3100**.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the

United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1	Full Name: Achim H. Krotz	Inventor's Signature: 	Date: 4/15/98
	Residence: Irvine, California	Citizenship: U.S.	
	Post Office Address: 26 Pergola Irvine, California 92715		
2	Full Name: Vasulinga T. Ravikumar	Inventor's Signature: 	Date 4/15/98
	Residence: Carlsbad, California	Citizenship: India	
	Post Office Address: 6606 Vireo Court Carlsbad, California 92009		
3	Full Name:	Inventor's Signature:	Date
	Residence:	Citizenship:	
	Post Office Address:		

DOCKET NO. ISIS-2710

PATENT



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:

Achim H. Krotz and Vasulinga T. Ravikumar

Serial No.: 09/032,972

Group Art Unit: 1801

Filed: January 26, 1998

Examiner: Not Yet Assigned

For: IMPROVED PROCESS FOR
SYNTHESIS OF OLIGONUCLEOTIDES

Assistant Commissioner for Patents
Washington DC 20231

Sir:

ASSOCIATE POWER OF ATTORNEY

The undersigned, of the firm WOODCOCK WASHBURN KURTZ
MACKIEWICZ & NORRIS LLP, One Liberty Place - 46th Floor, Philadelphia, Pennsylvania
19103, Attorney and/or Agents for Applicant(s), hereby appoints the following:


Robert B. Washburn	Registration No. 16,574	David R. Bailey	Registration No. 35,057
Richard E. Kurtz	Registration No. 19,263	Barbara L. Mullin	Registration No. 38,250
John J. Mackiewicz	Registration No. 19,709	Lynn A. Malinoski	Registration No. 38,788
Norman L. Norris	Registration No. 24,196	Lori Y. Beardell	Registration No. 34,293
Albert W. Preston, Jr.	Registration No. 25,366	Doreen Yatko Trujillo	Registration No. 35,719
Dale M. Heist	Registration No. 28,425	Paul K. Legaard	Registration No. 38,534
Philip S. Johnson	Registration No. 27,200	Kevin M. Flannery	Registration No. 35,871
John W. Caldwell	Registration No. 28,937	David A. Cherry	Registration No. 35,099
Gary H. Levin	Registration No. 28,734	Anthony J. Rossi	Registration No. 24,053
Steven J. Rocci	Registration No. 30,489	Michael J. Swope	Registration No. 38,041
Dianne B. Elderkin	Registration No. 28,598	Michael J. Bonella	Registration No. P41,628
Francis A. Paintin	Registration No. 19,386	Harold H. Fullmer	Registration No. P42,560
John P. Donohue, Jr.	Registration No. 29,916	John E. McGlynn	Registration No. P42,863
Henrik D. Parker	Registration No. 31,863	Gail Ann Dalickas	Registration No. 40,979
Suzanne E. Miller	Registration No. 32,279	Kimberly R. Hild	Registration No. 39,224
Lynn B. Morreale	Registration No. 32,842	Lawrence A. Aaronson	Registration No. 38,369
Mark DeLuca	Registration No. 33,229	Jonathan M. Waldman	Registration No. 40,861
Joseph Lucci	Registration No. 33,307	Joseph R. Condo	Registration No. P42,431
Michael P. Dunnam	Registration No. 32,611	Michael K. Jones	Registration No. P41,100
Michael D. Stein	Registration No. 34,734		
Albert J. Marcellino	Registration No. 34,664		

and

Herb Boswell Registration No. 27,311
Laurel Bernstein Registration No. 37,280
Andrew E. Granston Registration No. 38,473

of **ISIS PHARMACEUTICALS, INC.**, 2292 Faraday Avenue, Carlsbad, California 92008;
his/her associates with full power to prosecute the above-identified application and to transact
all business in the Patent Office connected therewith and requests that correspondence
continue to be directed to the firm of **WOODCOCK WASHBURN KURTZ MACKIEWICZ
& NORRIS LLP** at the above address.

Date: June 22, 1998



Michael P. Straher
Registration No. 38,325

Woodcock Washburn Kurtz
Mackiewicz & Norris LLP
One Liberty Place - 46th Floor
Philadelphia PA 19103
Telephone: (215) 568-3100
Facsimile: (215) 568-3439

© 1997 WWKMN

Applicant or Patentee: Achim H. Krotz and Vasulinga T. Ravikumar

Serial No.: To Be Assigned

Attorney's Docket No.: ISIS-2710

Date Filed: Herewith

For: IMPROVED PROCESS FOR SYNTHESIS OF OLIGONUCLEOTIDES

**VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27(c) - SMALL BUSINESS CONCERN)**

I hereby declare that I am:

() the owner of the small business concern identified below:

(X) an official empowered to act on behalf of the concern identified below:

NAME OF CONCERN: ISIS Pharmaceuticals, Inc.

ADDRESS OF CONCERN: 2292 Faraday Avenue
Carlsbad, CA 92008

I hereby declare that the above-identified small business concern qualifies as a small business concern as defined in 13 CFR 121.12, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, in that: (1) the number of employees of the concern, including those of its affiliates, does not exceed 500 persons; and (2) the concern has not assigned, granted, conveyed, or licensed, and is under no obligation under contract or law to assign, grant, convey, or license, any rights in the invention to any person who could not be classified as an independent inventor if that person had made the invention, or to any concern which would not qualify as a small business concern or a nonprofit organization under this section. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention entitled **IMPROVED PROCESS FOR SYNTHESIS OF OLIGONUCLEOTIDES** by inventor(s) **Achim H. Krotz and Vasulinga T. Ravikumar** described in

(X) specification filed herewith.

RECEIVED 22520000

SCANNED 7

() application serial no. _____, filed _____

() patent no. _____, issued _____.

If the rights held by the above-identified small business concern are not exclusive, each individual, concern or organization having rights in the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 CFR 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d), or a nonprofit organization under 37 CFR 1.9(e).

***NOTE: Separate verified statements are required for each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)**

FULL NAME:

ADDRESS:

() INDIVIDUAL () SMALL BUSINESS CONCERN () NONPROFIT ORGANIZATION

FULL NAME:

ADDRESS:

() INDIVIDUAL () SMALL BUSINESS CONCERN () NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING
TITLE OF PERSON SIGNING
ADDRESS OF PERSON SIGNING

Daniel L. Kisner
President & Chief Operating Officer
2292 Faraday Avenue
Carlsbad, CA 92008

D. Kisner MD
SIGNATURE

2/25/98
DATE